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An oestrogen receptor β-selective agonist exerts anti-neoplastic effects in experimental intrahepatic cholangiocarcinoma

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ABSTRACT

Background: Cholangiocarcinoma cells over-express oestrogen receptor-β, which displays anti-proliferative and pro-apoptotic effects.
Aim: To evaluate the effects of a newly developed and highly selective oestrogen receptor-β agonist (KB9520) on experimental intrahepatic cholangiocarcinoma.
Methods: In vitro, the effects of KB9520 on apoptosis and proliferation of HuH-28 cells, HuH-28 cells with selective oestrogen receptor-β silencing (by small interfering RNA), HepG2 cells (oestrogen receptor-α and oestrogen receptor-β negative) and HepER3 cells (HepG2 cells transformed to stably express oestrogen receptor-α) were evaluated. *In vivo*, the effects of KB9520 on experimental intrahepatic cholangiocarcinoma, induced by thioacetamide administration were tested.
Results: In vitro, KB9520 induced apoptosis and inhibited proliferation of HuH-28 cells. KB9520 effects were absent in cells lacking oestrogen receptor-α and β (HepG2) and in cells expressing only oestrogen receptor-α (HepER3); its pro-apoptotic effect was impaired in cells where oestrogen receptor-β expression was decreased by specific small interfering RNA. *In vivo*, KB9520 inhibited experimental intrahepatic cholangiocarcinoma development in thioacetamide-treated rats and promoted tumour regression in rats where tumour was already established. In treated animals, tumour areas showed reduced proliferation but increased apoptosis.
Conclusions: KB9520 induced apoptosis in cholangiocarcinoma by selectively acting on oestrogen receptor-β, suggesting that oestrogen receptor-β selective agonists may be a novel and effective therapeutic option for the medical treatment of intrahepatic cholangiocarcinoma.

1. Introduction

Intrahepatic cholangiocarcinoma (CCA) is a devastating and highly aggressive cancer with an overall 5 year survival rate of less than 10% after diagnosis [1–4]. There is no effective pharmacological treatment since CCA cells show high resistance to chemotherapeutic agents [1]; thus, there is an urgent need to develop novel therapeutic approaches.

Liver biopsies from patients with intrahepatic CCA were strongly positive for both oestrogen receptor (ER)-α and ER-β subtypes in 100% of examined patients, whilst cholangiocytes from normal liver were negative [5,6]. Compared to benign cholangiocyte proliferation, intrahepatic CCA cells showed a higher expression of ER-α and ER-β [5–7] with an enhanced ER-α/ER-β ratio. This observation is in agreement with many different reports showing an increased ER-α/ER-β ratio in cancerous versus normal tissues, including ovary, prostate, colon and breast cancers [8–11]. In these tissues, primary events in neoplastic transformation and progression have been correlated with up-regulation of ER-α and down-regulation of ER-β, which mainly occurs during adenoma–carcinoma transition, thus,

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associating the function of ER-α subtype with carcinogenesis rather than cancer cell proliferation [8–11]. Recently, we have shown that ER-α acts synergistically with the IGF1 axis in promoting CCA cell proliferation and also that this receptor subtype stimulates VEGF synthesis and neoangiogenesis [5,6]. The opposite was suggested for ER-β, which has been reported to have a protective effect against aberrant cell proliferation and carcinogenesis [12]. Genetic or pharmacological strategies aiming to induce expression or activation of ER-β are currently under investigation for cancer prevention or treatment [12]. Since, in contrast to other cancers, high ER-β expression is maintained in intrahepatic CCA even at late stages [5], it can be hypothesized that selective activation of ER-β may be beneficial to limit tumour growth.

Aim of this study was to evaluate *in vitro* and *in vivo* the effects of a newly developed and highly selective ER-β agonist (KB9520) on experimental intrahepatic CCA.

2. Methods

2.1. Materials

Reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated. Media and serum for cell culturing were obtained from Life Technologies, Inc. (Gaithersburg, MD). KB9520 [13] was synthesized and provided by Karo Bio (Rhönnstad P, Kallin E, Apelqvist T et al. Novel estrogen receptor ligands. Patent application # WO2009/127686, 22 October 2009). ICI 182,780 was purchased from Tocris Bioscience. ER-β antibodies were purchased from Santa Cruz Biotechnologies Inc. (Santa Cruz, CA), unless otherwise indicated. CK-19 antibodies were obtained from Novocastra (Milan, Italy) and caspase 3 antibodies were purchased from Cell Signalling (Boston, MA).

2.2. Cell lines

The HuH-28 cell line (derived from intrahepatic CCA) was obtained from Cancer Cell Repository, Tohoku University, Tohoku, Japan [5]. HepG2 cells (ATCC no. HB-8065), is a human hepatocarcinoma cell line negative for ER-α and ER-β, and HepER3 cells are HepG2 cells transformed to stably express the human ER-α [14] (provided by Karo Bio AB Huddinge, Sweden). HepG2 and HepER3 cells, were seeded on 6-well plates and maintained in MEM (Bio-Concept's AMIMED, Switzerland) supplemented with 10% foetal bovine serum (FBS), 1% penicillin 100 U/ml, 100 mg/ml streptomycin, 1% L-glutamine 200 mM (GIBCO), 1%NEAA, 1% Na-pyruvate, 1% L-glutamine in a humidified atmosphere of 5% CO_2. The different cell lines were exposed to the ER-β-selective agonist, KB9520 (prepared as a DMSO stock solution) was diluted to the indicated concentrations with a final maximal DMSO dilution of 1:1,000,000. DMSO was also present in control medium (dilution = 1:1,000,000). Media and additives for cell culture were obtained from Gibco (BRL, Invitrogen Corporation) unless otherwise indicated.

2.3. Isolated bile duct units (IBDU) from human liver

Fragments of intrahepatic bile ducts, averaging 20 μm in diameter, were isolated from human liver (4 liver donors, 1 g pieces) as previously described [15], exposed to increasing concentration (from 0.1 to 1000 nM) of KB9520 and apoptosis was evaluated by measuring caspase 3 activity and terminal deoxynucleotidyl transferase-mediated triphosphate end-labelling (TUNEL) positive cells.

2.4. Apoptosis assays

Apoptosis was evaluated by caspase-3 activity or TUNEL staining [5,6]. Caspase-3 activity was determined by colorimetric assay kit (Sigma–Aldrich), based on the hydrolysis and release of pNA from the peptide substrate acetyl-Asp-Glu-Val-Asp-p-nitroanilide (Ac-DEVD-pNA). Cells were lysed in lysis buffer provided by the vendor and the concentration of caspase-3 mediated release of pNA was determined spectrophotometrically at 405 nM and normalized for protein concentration. Caspase 3 activity is expressed as % change from DMSO control. For TUNEL staining determination the DeadEnd™ Colorimetric TUNEL System (Promega, Madison, WI) was used according to the supplier's recommendation. This method is used to detect DNA fragmentation in cells undergoing apoptosis by the incorporation of a biotinylated nucleotide at the 3′-OH end of fragmented DNA. Results are expressed as number of TUNEL positive cells per 100 counted cells. For apoptosis evaluation, conditioned cell medium supplemented with 10% foetal serum was replaced with fresh medium containing the test compound and a final dilution of DMSO of 1:1,000,000. Control cells were incubated with fresh medium containing DMSO only (final DMSO dilution = 1:1,000,000). After 72 h incubation, apoptosis activity was measured by the Caspase 3 or TUNEL methods.

2.5. Silencing of ER-β in HuH-28 cell by small interfering RNA (siRNA)

Duplexed RNA oligonucleotides (Stealth RNAi); ESR2HSS103380 (SEQ 1), ESR2HSS103378 (SEQ 2) and ESR2HSS176622 (SEQ 3) were synthesized by Invitrogen and used to knock down the expression of ER-β in HuH-28 cells. As a control, we used Stealth RNAi negative control (scrambled) duplexes (Invitrogen). The efficacy of siRNA transfections was determined according to the manufacturer's instructions by using Stealth RNAi glyseraldehyde-3-phosphate dehydrogenase (GAPDH) positive control duplexes (Invitrogen). HuH-28 cells were cultured in 12-well plates and used at 50–70% density the day of transfection. Cells were transfected with Lipofectamine RNAiMAX (Invitrogen) according to manufacturer's instruction. Following siRNA treatment (72 h), HuH-28 cells were harvested and assessed for ER-β protein levels by western blotting and the apoptotic effect of KB9520 was evaluated in comparison with control cells treated with scrambled RNA.

2.6. Western blot

Cells were harvested, washed with PBS and whole cell lysate was prepared on ice by suspending the cells in 100–150 μL of RIPA-Buffer containing 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 2 μg/ml aprotinin. Protein concentration was determined with the Bio-Rad Protein Assay-Dye Reagent (Bio-Rad Laboratories GmbH, Germany). For immunoblotting, 30 μg total protein per extract was separated on SDS-PAGE, electro-transferred onto nitrocellulose membranes, and probed with anti-ER-β monoclonal antibody and anti-β-actin mouse monoclonal antibody (Sigma Chemical). Detection of specific proteins was carried out with an enhanced chemiluminescence western blotting kit (Qdot Western blotting kit, Invitrogen).

2.7. Proliferation assay

Cell proliferation was assessed by the MTS assay (Cell Titer 96 AQueous Non-Radioactive Cell Proliferation Assay, MTS Kit, Promega, Madison, WI) according to the manufacturer's instructions. Absorbance values were measured at 490 nm after exposure of cells to 20 μl MTS reagent for 2 h. Proliferation index was cal-